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Association of Mutations in the Basal Core Promoter and Pre-core Regions of the Hepatitis B Viral Genome and Longitudinal Changes in HBV Level in HBeAg Negative Individuals: Results From a Cohort Study in Northern Iran

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Background: Although certain HBV mutations are known to affect the expression of Hepatitis e antigen, their association with HBV viral level or clinical outcomes is less clear.

Objectives: We evaluated associations between different mutations in the Basal Core promoter (BCP) and Pre-core (PC) regions of HBV genome and subsequent changes in HBV viral DNA level over seven years in a population of untreated HBeAg negative chronic hepatitis B (CHB) participants in Northeast of Iran.

Materials and Methods: Participants in the current study were drawn from the Golestan Hepatitis B Cohort Study (GHBCS), a cohort of approximately 2590 HBsAg positive subjects (living in Gonbad city) embedded in the Golestan Cohort Study (GCS). At baseline, HBsAg was measured in all participants and revealed 2590 HBsAg positive cases. We randomly selected 304 participants who their blood sample were taken at both baseline and seven years later in follow-up and had not been treated for HBV during this time. HBV viral load were assessed at baseline and at year 7. The BCP and PC regions of the HBV DNA, at baseline, were amplified via hemi-nested PCR and sequenced by cycle sequencing. At year 7, liver stiffness was assessed by fibroscan; also, other parameters of liver disease were assessed following standard clinical protocols. Associations were assessed via tabulation, chi-square, t-tests and logistic regression. P values < 0.05 were considered statistically significant and all tests were two-sided.

Results: Among 304 HBsAg positive participants, 99 had detectable HBV DNA at study baseline. Of these, 61.6% had PC mutations (48.5% A1896 and 25.2% G1899). In contrast to other mutations, A1896 was associated with a higher proportion of detectable HBV DNA at year 7 (39.6%) compared to patients with the wild type (13.7%) (OR: 4.36, CI95% = 1.63-11.70; P Value = 0.002). Although participants with the A1896 mutation had higher year-7 HBV viral load than participants with G1896 (2.30 ± 1.66 IU/mL vs. 1.76 ± 1 IU/mL among patients with detectable HBV; P value = 0.052), no association was observed with either serum level ALT or liver stiffness. Interestingly, mutations in the basal core promoter (BCP) region had no significant effect on virus DNA detection.

Conclusions: In this population with chronic HBeAg negative hepatitis B, an association was observed between the G1896A mutation in the Pre-core region of HBV and subsequent level of HBV DNA seven years later, which indicated that mutations in this region of HBV genome may contribute to disease progression in these patients and play an important role in HBV natural course of disease.

Keywords: Hepatitis B, Chronic; Mutation; Genome, Viral

1. Background

It is estimated that two billion people are infected with Hepatitis B virus (HBV) and 350 million people have chronic HBV infection (CHB) in the world (1). Nearly 75% of infected individuals live in Asian countries (2). Most patients infected with acute HBV undergo a three-step process: immune tolerance, immune clearance and seroconversion. However, a number of patients develop a fourth, "HBeAg negative" phase (3), in which patients have negative results for the Hepatitis e antigen (HBeAg), but have detectable HBV viral DNA in the blood. Although initially considered a late phase of infection that was manifested in the elderly, this pattern has subsequently been reported to occur in an increasing proportion of cases worldwide. For example, HBeAg-negative CHB is the most common type of chronic hepatitis B in the European, African and Middle East countries of the Mediterranean Basin (3).

HBV genotype and HBV DNA levels are among the most important predictors of long-term clinical outcomes (4). However, specific mutations in HBV may also be important. HBV experiences evolutionary selection, with an estimated rate of nucleotide substitution of approximately 1.4 to 3.2 × 10^{-5} per site per year, reflecting the error rate

of the viral reverse transcriptase (4). Such mutations may alter interactions with host immune system, as well possibly potentiate viral replication, enhance virulence and promote resistance to antiviral therapies (4).

Mutations can occur throughout the HBV genome, including regions such as the pre-core, core promoter and pre-S/S genes (5), critical to the HBV function (6). Mutation of pre-core nucleotide at position 1896 from guanine (G) to adenine (A) and changes of two other nucleotides, an adenine (A) to thymine (T) transversion at nucleotide 1762 together with a guanine (G) to adenine (A) transition at nucleotide 1764 within the basal core-promoter (BCP) have been shown to prevent the synthesis of HBeAg (7). These mutations are often observed in HBeAg-negative patients with active viral replication and liver disease. Asymptomatic hepatitis B carriers sometimes have these mutations too. Double mutations in BCP T1762/A1764 have been shown to increase the risk of liver disease progression and hepatocellular carcinoma (HCC) within HBV genotypes B and C (4). Less data is available for other viral genotypes. In addition, clinical and mechanistic importance of pre-core mutations are unclear; especially G1896A, which results in a premature stop codon and termination of HBeAg translation, but does not seem to affect viral replication. Studies in population-based cohorts are particularly lacking (8, 9)

Relative to other parts of the world, the prevalence of HBV in Iran varies from low to intermediate based on geographic region. Although there are an estimated 1.5 million chronically infected individuals (5), the rate of liver cancer is lower than might be expected, perhaps explained by a higher prevalence of genotype D, and high prevalence of HBeAg seronegativity or because of some mutations in core or pre-core region of the virus (10, 11). On the other hand, HBV positive individuals in Middle Eastern countries have been shown to have a high susceptibility for acquisition of pre-core or basal core promoter variants that may affect HBeAg (10), but perhaps not HBV replication or other aspects of HBV. Within Iran, there exists strong geographic variation. Whereas, the prevalence of HBV is intermediate-to-high in the Golestan province of northern Iran (12, 13), the prevalence is far lower in nearly every other province (1).

2. Objectives

In the current analysis, we conducted a longitudinal study to assess the association of mutations in the BCP and PC regions of the HBV viral genome with subsequent levels of HBV viral DNA and severity of liver disease in a cohort of untreated HBeAg negative, genotype D, chronic hepatitis B patients followed for a long time.

3. Materials and Methods

3.1. Study Population

Our study population was a subset of the Golestan Cohort Study (GCS), initiated in 2004 in Northern Iran (14-17). As previous data suggested a high prevalence of HBV infection in this region (13), we created the Golestan Hepatitis B Cohort Study (GHBCS) from the subset of GCS participants who lived in Gonbad city (n = 9559). All 9559 of these participants were measured for hepatitis B surface antigen (HBsAg) at baseline, and 2590 participants were found to have positive results. Each participant donated a second blood sample after seven years of follow-up.

For the current analysis, we included a random sample of 394 chronically positive patients (HBsAg + longer than six months) who lacked the concomitant hepatitis C or D, human immune deficiency virus (HIV) infection, or evidence of autoimmune hepatitis, Wilson's disease or primary biliary cirrhosis. These participants were then tested for HBeAg. We excluded five HBeAg positive cases and 85 participants who lacked a second collected blood sample or who were otherwise lost to follow-up. Then, we measured HBV DNA in the remaining 304 participants, subsequently excluding 205 participants with undetectable HBV DNA at baseline. DNA samples from these 99 participants were sent for sequencing of the Basal Core Promoter (BCP) and pre-core (PC) regions of the HBV viral genome.

All included participants were HBsAg positive at both baseline and year 7. None of them received treatment during the follow-up. Informed consent was obtained and our study was approved by the ethical committee of the Digestive Disease Research Institute (DDRI) and registered in the research deputy of TUMS (No.92-02-37-19250).

Remaining in the study and follow-up were all voluntarily. Names and tests results were all confidential and just handed to the cases themselves.

3.2. Laboratory Testing

Baseline and year 7 serum samples were stored at -80°C until use. Serologic markers for HBV HBsAg, antibody to hepatitis B surface antigen (anti-HBs), antibody to hepatitis B core (anti-HBc), HBeAg, and anti-HBe) were assessed using a commercially available ELISA kit from RADIM (Roma Rome, Italy). These tests along with HBV DNA level measurements were performed at both baseline and year 7 samples. A biochemical panel was performed on all fresh samples at year 7 consisted of Alanine transaminase (ALT), Aspartate transaminase (AST), gamma-glutamyl transferase (GGT),Complete Blood Count (CBC), Fasting Blood Sugar (FBS) and lipid profile.

3.3. HBV-DNA Quantification

HBV DNA was extracted from 200 μL of serum using QIAamp DNA Blood Mini Kit (QIAGEN, Valencia, CA, USA) and then quantified in the Light-Cycler (Roche Diagnostics, Mannheim, Germany) by RealArtTM HBV LC PCR (QIAGEN, Hilden, Germany) according to the manufacturer's instructions. The linear range of this assay was 10^2 - 10^9 copies/mL. For our main analysis, we classified participants as having either detectable or undetectable HBV viral DNA in year 7 of follow-up.